DIFFERENCES IN ENZYME CONTENT OF AZUROPHIL AND SPECIFIC GRANULES OF POLYMORPHONUCLEAR LEUKOCYTES

II. Cytochemistry and Electron Microscopy of Bone Marrow Cells

DOROTHY FORD BAINTON and MARILYN G. FARQUHAR

From the Department of Pathology, University of California School of Medicine, San Francisco, California 94122

ABSTRACT

In the previous paper we presented findings which indicated that enzyme heterogeneity exists among PMN leukocyte granules. From histochemical staining of bone marrow smears, we obtained evidence that azurophil and specific granules differ in their enzyme content. Moreover, a given enzyme appeared to be restricted to one of the two types. Clear results were obtained with alkaline phosphatase, but those with a number of other enzymes were suggestive rather than conclusive. Since the approach used previously was indirect, it was of interest to localize the enzymes directly in the granules. Toward this end, we carried out cytochemical procedures for five enzymes on normal rabbit bone marrow cells which had been fixed and incubated in suspension. The localization of reaction product in the granules was determined by electron microscopy. In accordance with the results obtained on smears, azurophil granules were found to contain peroxidase and three lysosomal enzymes: acid phosphatase, arylsulfatase, and 5'-nucleotidase; specific granules were found to contain alkaline phosphate. Specific granules also contained small amounts of phosphatasic activity at acid pH. Another finding was that enzyme activity could not be demonstrated in mature granules with metal salt methods (all except peroxidase); reaction product was seen only in immature granules. The findings confirm and extend those obtained previously, indicating that azurophil granules correspond to lysosomes whereas specific granules represent a different secretory product.

INTRODUCTION

In the preceding paper (1) we reported observations, based on histochemical staining of bone marrow smears, which indicate that differences exist in the enzyme content of azurophil and specific granules of PMN (polymorphonuclear) leukocytes. In this paper we report the results of tests for five enzymes (peroxidase, acid phosphatase, arylsulfatase, 5'-nucleotidase, and alkaline phosphatase) performed on bone marrow cells, fixed and incubated in suspension, and examined by electron microscopy. The present results, already reported in part elsewhere (2), support the

previous findings on the existence of enzyme heterogeneity among PMN granules by demonstrating directly differences in the enzyme content of the two granule types. In contrast to the situation encountered previously in smears, however, when metal salt techniques are applied to cells fixed and incubated in suspension, the enzyme activity in mature PMN granules is latent and only immature forms are reactive.

MATERIALS AND METHODS

Materials

Observations were made on PMN leukocytes from bone marrow of 35 young adult New Zealand rabbits.

Substrates were obtained from Sigma Chemical Co., St. Louis, Mo., as follows: β-glycerophosphate (types I, II, III), cytidine 5'-monophosphate (disodium salt), adenosine-5'-monophosphate (type II), 3,3'-diaminobenzidine tetrahydrochloride, and p-nitrocatechol sulfate.

Techniques for Cytochemical Studies

COLLECTION AND FIXATION OF BONE MARROW CELLS: Femoral marrow was collected and fixed in suspension in 15-ml conical tubes as described previously (3). Fixation was carried out in one of the following solutions: (a) 1.5% distilled (4) glutaraldehyde (5) in 0.1 M sodium cacodylate-HCl buffer (pH 7.4) with 1% sucrose for 10–60 min at 4° or 25° C; (b) formol-calcium, pH 7.4, (6) freshly prepared from paraformaldehyde (7), for 60 min at 4°C; (c) paraformaldehyde-glutaraldehyde, (8) diluted 1:1 with 0.2 M cacodylate-HCl buffer for 30–60 min at 25° C. After fixation the cells were spun at 3,000 rpm, the fixative was decanted, and the cells were resuspended by gentle pipetting. They were then washed three times (by repeated spinning and resuspension) in 0.1 M cacodylate-HCl buffer with 7% sucrose (pH 7.4) at 4° C. After the last wash, cells were spun and stored at 4° C from 10 min to 24 hr. With specimens fixed in formol-calcium, washing and storage were carried out in 7% sucrose.

INCUBATION: Incubation media were prepared as described below, and 5% sucrose was added (9) to all except the dye media. Cells were resuspended by gentle pipetting until they were uniformly distributed and incubated with continuous shaking for 10–90 min at 37° C, except in the case of the peroxidase tests which were carried out at 25° C.

AZO DYE METHODS: Cells fixed in formol-calcium were incubated for acid and alkaline phosphatase and arylsulfatase in the dye media described in Table I of the preceding paper (1).

PEROXIDASE METHOD: Unfixed cells and those fixed in glutaraldehyde or paraformaldehyde-glutaraldehyde were incubated at pH 7.6 in the diaminobenzidine medium of Karnovsky (10, 11) with H_2O_2 as substrate. Controls consisted of incubations in which 0.01 M KCN was added to the medium or in which diaminobenzidine or H_2O_2 was omitted.

ARYLSULFATASE METHOD: Incubations of glutaraldehyde-fixed cells were carried out at pH 5.5 in media prepared according to Goldfischer (12) or Hopsu-Havu et al. (13), with p-nitrocatechol sulfate as substrate and lead or barium, respectively, as capture agents. Some of the cells incubated in Goldfischer's medium were treated with $(NH_4)_2S$ to convert lead sulfate to lead sulfide (see reference 14). Cells were washed five times in 0.05 M acetate-Veronal buffer (pH 7.4) with 7% sucrose and subsequently were resuspended in 2% $(NH_4)_2S$ in the same buffer for 5 min. Controls consisted of incubations in which the substrate was omitted.

5'-NUCLEOTIDASE METHOD: Glutaraldehyde-fixed cells were incubated at pH 4.0 in Wachstein and Meisel's medium (15), with adenosine-5'-monophosphate as substrate and lead as capture agent. Controls consisted of incubations in which 0.01 M NaF was added to the medium, or the substrate was omitted.

ALKALINE PHOSPHATASE METHOD: The following four different procedures, using β-glycerophosphate as substrate at pH 9.0, were tried on glutaraldehyde-fixed tissue: (a) the original calcium method of Gomori (16), (b) the lead method of Hugon and Borgers (17), (c) the cadmium method of Mizutani and Barrnett (18), and (d) a method involving conversion of calcium to lead phosphate (19, 20). Controls consisted of incubations in which the substrate was omitted or 0.001 M cysteine was added to the media.

ACID PHOSPHATASE METHOD: Tests were performed on unfixed cells and those fixed in glutaraldehyde, paraformaldehyde-glutaraldehyde, or formol-calcium in media prepared according to Gomori (16) or Barka and Anderson (21), with β-glycerophosphate and lead at pH 5.0. Some incubations were also carried out at pH 5.0 with cytidine monophosphate as substrate (22). Controls were carried out adding 0.01 M NaF to the medium or by omitting the substrate.

SUBSEQUENT PROCESSING: After incubation, cells were washed three times by slow centrifugation and resuspension in 0.05 M acetate-Veronal buffer (pH 7.4) containing 7% sucrose. Some cells incubated by each method were processed for light microscopy, and, except in the case of azo dye procedures, some were processed for electron microscopy. For light microscopy, whole mounts were prepared on glass slides and counterstained as described for smears in the preceding paper (1). All except the preparations incubated for peroxidase were treated with dilute $(NH_4)_2S$ prior to mounting.

For electron microscopy, cells were placed in 0.4-ml polyethylene tubes and packed by centrifugation at
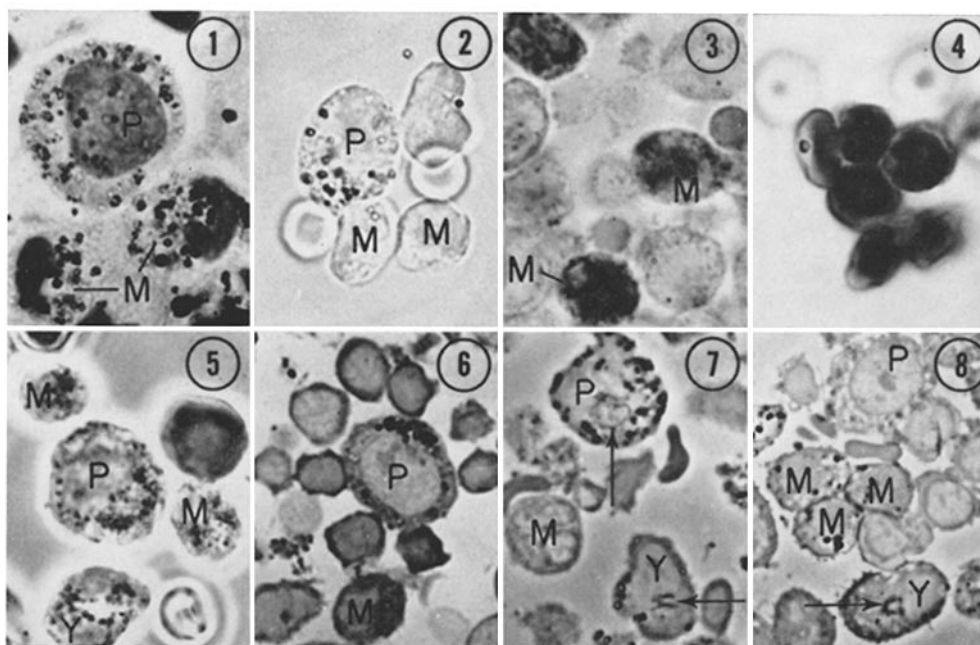
For Figs. 1–8 the following abbreviations are used: P, progranulocyte; M, mature PMN, band cell, or metamyelocyte; Y, myelocyte. Figs. 1–4 and 6 are bright-field, and Figs. 5, 7, and 8 are phase photomicrographs of cells from normal rabbit bone marrow. Fig. 1 is from a smear preparation; Figs. 2–5 are from whole mounts; and Figs. 6–8 are from 1 μ sections of cells fixed and incubated in suspension. Figs. 1–8, $\times$ 1320.

FIGURES 1–2 Preparations incubated for 5'-nucleotidase, showing the difference in reactivity of smears (Fig. 1) and whole mounts (Fig. 2) with metal salt methods. Note that cells in the smear are larger and more reactive for a given stage; many reactive granules are seen at all stages, with the progranulocyte (P) containing the greatest number. In the whole mount, fewer reactive granules are present in the progranulocyte (P), and none are seen in mature cells (M). Specimens fixed in glutaraldehyde, incubated 90 min at 37° C inWachstein-Meisel's medium (pH 4.0) with adenosine-5'-monophosphate, treated with (NH₄)₂S, counterstained with hematoxylin, and mounted in Piccolyte.

FIGURES 3–4 Whole mounts reacted for alkaline phosphatase with azo dye (Fig. 3) or metal salt (Fig. 4) methods. An intense reaction is evident in the mature cells (M) in Fig. 3, but no reaction is seen in those in Fig. 4. Specimens fixed in formol-calcium. The cells in Fig. 3 were incubated 30 min at 25° C in Burstone's medium and mounted in glycerogel. Those in Fig. 4 were incubated 2 hrs at 37° C in Gomori's calcium medium (pH 9.2), treated with lead nitrate (20) and (NH₄)₂S, counterstained with hematoxylin, and mounted in Piccolyte.

FIGURE 5 Whole mount, incubated for peroxidase. Numerous reactive granules are seen at all developmental stages indicated, with progranulocytes (P) containing the greatest number. Specimens fixed in glutaraldehyde, incubated 30 min at 25° C in Karnovsky's medium, and mounted in glycerogel.

FIGURE 6 Section of cells incubated for 5'-nucleotidase; field similar to that in the whole mount shown in Fig. 2. Specimens fixed and incubated as in Fig. 2, postfixed in OsO₄, treated in block with uranyl acetate, and embedded in Araldite. Section treated with (NH₄)₂S and stained with crystal violet.

FIGURES 7–8 Sections of cells incubated for acid (Fig. 7) or alkaline (Fig. 8) phosphatase. In Fig. 7, a number of reactive granules are present in the progranulocyte (P) and a few in the myelocyte (Y), but none are recognizable in the mature cell (M). In Fig. 8, none of the granules in the progranulocyte (P) are reactive, but some are seen in the myelocyte (Y), and a few in mature cells (M). Reaction can also be seen in the Golgi region (arrows) of the progranulocyte and myelocyte in Fig. 7 and in the myelocyte in Fig. 8. Specimens prepared as in Fig. 6, except that in Fig. 7 the specimen was incubated for 90 min at 37° C in modified Gomori's medium with β-glycerophosphate at pH 5.0; that in Fig. 8 was incubated for the same interval in Gomori's medium at pH 9.2 followed by treatment with lead nitrate.

~ 10,000 g for 4 min with a Microfuge 152 (Beckman Instruments, Inc., Spinco Division, Palo Alto Calif.), as suggested by Malamed (23). Subsequently the tube was cut open, and the small pellet of cells was removed and cut into 1 X 1 mm blocks. These were postfixed for 1 hr at 4° C in 1% OsO₄ in acetate-Veronal buffer (pH 7.4) with 5% sucrose added, treated with buffered 0.5% uranyl acetate containing 4% sucrose for 1 hr at room temperature (4, 24), dehydrated in graded ethanols, and embedded in Araldite or Epon (25). Some specimens were dehydrated directly after incubation or after postfixation in OsO₄.

Thin sections (∽ 500 A) which had been prepared with a Sorvall MT-2 microtome equipped with a diamond knife were examined either unstained or after staining with alkaline lead (26) or lead preceded by 6% aqueous uranyl acetate. In general, the most

satisfactory contrast was obtained by staining the section briefly (1–2 min) in lead citrate. Staining with uranyl acetate increases the density of the azurophil granules and thereby makes it difficult to recognize reaction product.

Sections $\frac{1}{2}$–2 μ thick were also prepared from the plastic blocks, affixed to glass slides by moderate heating, treated with 0.5–2% (NH₄)₂S for 45 min (4) (except in the case of specimens incubated for peroxidase), and lightly stained with crystal violet.

Microscopy

The "thick" Araldite ($\frac{1}{2}$–2 μ) or Epon sections were examined and photographed by light microscopy as described in the preceding paper (1).

Electron micrographs were taken at original magnifications of 3,000–30,000 with a Siemens Elmiskop I

Dense-cored Vacuole   Multicored Vacuoles   Multilobulated Form   Nucleoid   Mature Azurophil Granule

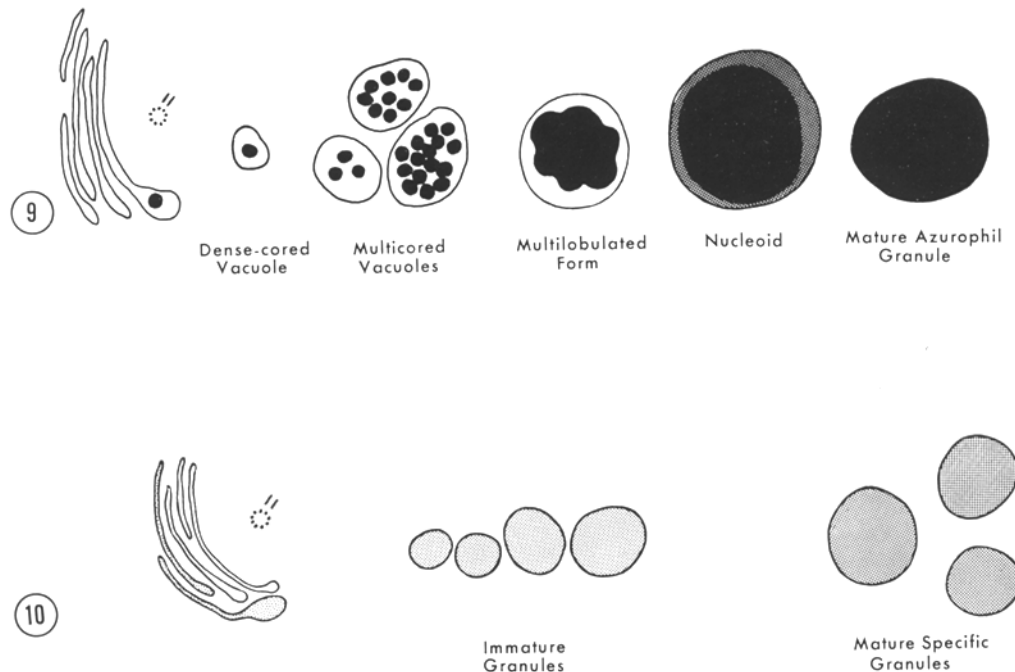Immature Granules   Mature Specific Granules

FIGURE 9 Diagrammatic representation of azurophil granule formation in PMN progranulocytes. Dense material condenses within the inner Golgi cisternae and pinches off to form a dense-cored vacuole (∽240 mμ). Several of the latter merge to form a multicored vacuole. The core material aggregates and becomes a multilobulated mass which subsequently forms a more compact nucleoid. The finely granular content around the core condenses until it is barely distinguishable from the core material, so that in the mature granule (diameter, 600–800 mμ) the entire content is uniformly dense.

FIGURE 10 Diagrammatic representation of specific granule formation in PMN myelocytes. Finely granular material condenses within the outer Golgi cisternae. Small granules (∽90 mμ) bud from the outer Golgi cisternae. Several of these merge to form larger aggregates which further condense to form mature granules, most of which are spherical and measure 300–500 mμ in diameter. A few dumbbell or smaller forms (not shown) are also present in mature PMN (see reference 3, Fig. 11).

TABLE I

Sites of Enzyme Reaction Product in Developing PMN

Enzyme test	Progranulocyte				Myelocyte					
	ER cisternae	Golgi cisternae	Immature azurophil granules	Mature azurophil granules	ER cisternae	Golgi cisternae	Immature specific granules	Mature specific granules	Immature* azurophil granules	Mature azurophil granules
Peroxidase	−	−	+	+	−	−	−	−	+	+
Arylsulfatase	+	+	+	−	−	−	−	−	+	−
5'-nucleotidase	−	−	+	−	−	−	−	−	+	−
Alkaline phosphatase	−	−	−	−	−	+	+	−	−	−
Acid phosphatase	+	+	+	−	+	+	+	−	+	−

* A few immature azurophil granules persist in the myelocyte.
− = no reaction seen
+ = reaction product present

operating at 80 kv with a double condenser and a 50 μ objective aperture.

Special Treatments

In an attempt to affect the acid phosphatase activity of mature granules, the following experiments were carried out on cells fixed for 30 min at 4° C in glutaraldehyde: (a) cells were subjected to repeated (six times) freezing in liquid nitrogen and thawing at 37° C prior to incubation or (b) preincubated in protamine (1 mg/ml) or citrate buffer (pH 2.5) for 30 min; (c) incubations were carried out in media with sparine (5 mM), protamine (1 mg/ml), Triton X-100 (0.5%), or DMSO (10%) added; (d) incubation times were prolonged to 3 hrs; or (e) instead of cells in suspension, 30-μ sections of bone marrow prepared on a cryostat or freezing microtome were used for incubation.

RESULTS

Light Microscopy

WHOLE MOUNTS COMPARED TO SMEARS: The cell size in whole mounts of cells fixed and incubated in suspension (Figs. 2–5) was smaller ($\sim\frac{1}{2}$) than that of cells in smears (Fig. 1) studied previously (1). The results with the peroxidase test (Fig. 5) and with the dye methods for arylsulfatase and acid and alkaline phosphatase (Fig. 3) were similar in both types of preparations. However, with the remaining enzyme tests, all of which involved metal salt methods, there were markedly fewer reactive granules for a given stage in the whole mounts (compare Figs. 1 and 2). This difference in the reactivity of the two types

of preparations was particularly evident in mature cells; those in whole mounts showed only occasional reactive granules with any metal salt method (Figs. 2 and 4). The marked reduction in reactivity of PMN, particularly mature PMN, incubated in suspension in metal salt media is explicable on the basis of the electron microscopic observations (see below) which indicate that only immature granules contain reaction product under these conditions. As far as the distribution of reactive granules during stages of PMN maturation is concerned, it was similar to that described previously for smears. With arylsulfatase, 5'-nucleotidase (Figs. 2 and 6), and acid phosphatase (Fig. 7), progranulocytes contained more than did later stages. With alkaline phosphatase, reactive granules were restricted to myelocytes and more mature forms (Fig. 8).

Electron Microscopy

The distribution of enzyme reaction products was determined within azurophil and specific granules and within cell organelles during stages of granule formation. We have previously shown (3) that the two types of PMN granules are produced in two separate generations; azurophil granules arise from the proximal or concave face of the Golgi complex of progranulocytes. Specific granules arise from the distal or convex face of the Golgi complex of myelocytes. The steps in granule formation and maturation are diagrammed in Figs. 9 and 10 and are referred to below.

Table I indicates the sites where enzyme reac-

tion products were localized in progranulocytes and myelocytes. The best results in terms of structural preservation and precise localization were obtained with glutaraldehyde-fixed tissue. Hence the descriptions that follow are based primarily on such tissue. However, with all the methods disruption of membranous organelles and extraction of granule contents occasionally were encountered. The distribution and intensity of the reaction product were the same in specimens fixed for 10 min at 25° C and for those fixed for up to 60 min at 4° C except in the case of peroxidase (see below), but tissue preservation was improved by longer fixation.

PEROXIDASE

GENERAL COMMENTS: Cells which were fixed for short periods (5–10 min) at 4° C showed a more intense reaction than those fixed at 25° C or for longer periods (60 min) at 4° C or those incubated without prior fixation.

LOCALIZATION: Reaction product was limited to azurophil granules where it was present in both immature and mature forms (Figs. 11–14). Among the immature granules, the earliest that consistently contained deposits was the multi-cored vacuole (Fig. 12). The reaction product appeared as a dense, flocculent precipitate localized at the granule periphery and filling the space

not occupied by the cores. Similar deposits were also seen consistently in multilobulated forms and nucleoids (Figs. 11 and 13). Among mature granules, most appeared uniformly dense, owing to the presence of reaction product throughout the granules (Figs. 11 and 14). Occasionally, the center of mature granules appeared lighter (Fig. 14), particularly in specimens fixed for longer periods. Reaction product was not seen in specific granules (Fig. 14) or in cisternae of the endoplasmic reticulum (ER) or Golgi complex of progranulocytes or myelocytes.

CONTROLS: No reaction was observed when diaminobenzidine was omitted. Similarly, a reaction was not usually seen when H_2O_2 was eliminated from the incubation medium except for rare extracted azurophil granules which showed deposits of reaction product at their periphery. Addition of 0.01 M KCN to the medium partially inhibited the reaction.

ARYLSULFATASE

GENERAL COMMENTS: The particles of reaction product, lead sulfate, frequently chipped out of the specimen (presumably during sectioning) leaving large holes in their place. When the tissue was treated after incubation with $(NH_4)_2S$ to convert lead sulfate into lead sulfide, the particles of

---

Abbreviations for Figs. 11–30:
G, Golgi complex
ag, mature azurophil granule
ce, centriole
er, rough-surfaced ER
ia, immature azurophil granule
ic, inner Golgi cisterna
is, immature specific granule
m, mitochondrion

mc, multicored vacuole
ml, multilobulated form
n, nucleus
oc, outer Golgi cisterna
pn, perinuclear cisterna
sg, mature specific granule
u, nucleoid
v, dense-cored vacuole

FIGURES 11–13 Developing PMN from preparations incubated for peroxidase. Fig. 11 shows part of a progranulocyte with the Golgi region (G) and part of the nucleus (n). Figs. 12 and 13 show immature azurophil granules at higher magnification. Reaction product is present in both immature and mature azurophils. Among immature granules, flocculent deposits denser than the core material are seen at the periphery of multicored (mc), multilobulated (ml), or nucleoid (u) forms. Mature granules (ag) appear uniformly dense due to the presence of reaction product throughout. Several granules show partially extracted centers (c). No reaction product is seen in single-cored vacuoles (v) or in cisternae of the ER (er) or Golgi complex (G). Specimens fixed in glutaraldehyde, incubated 30 min at 25° C in Karnovsky's medium, postfixed in OsO4, and embedded in Araldite or Epon. Sections stained with lead citrate. The specimen in Fig. 12 was treated in block with uranyl acetate prior to dehydration. Fig. 11, × 18,000; Fig. 12, × 28,000; Fig. 13, × 35,000.
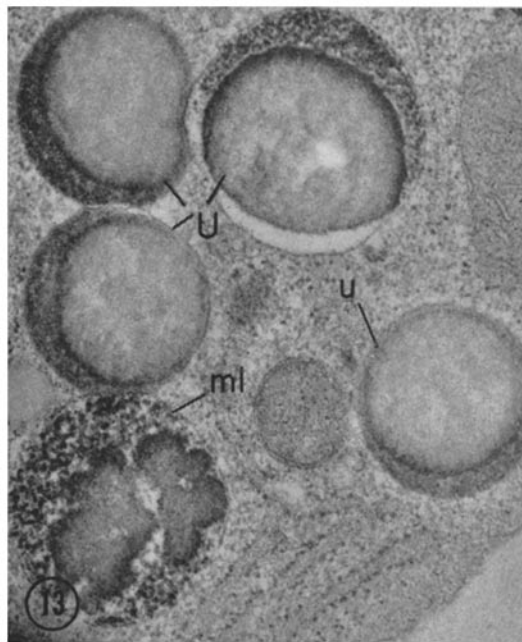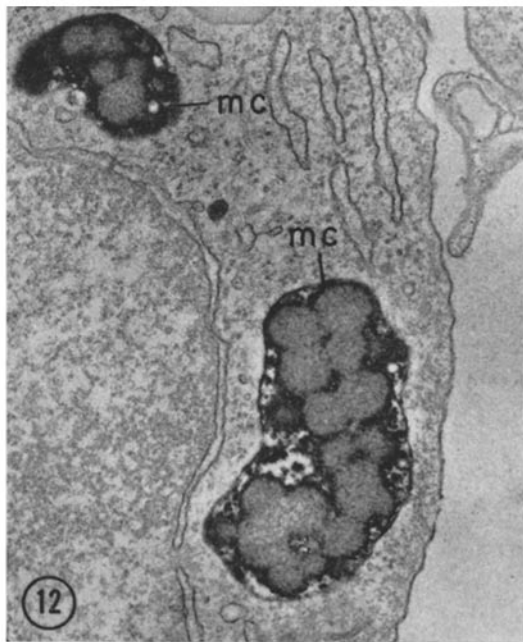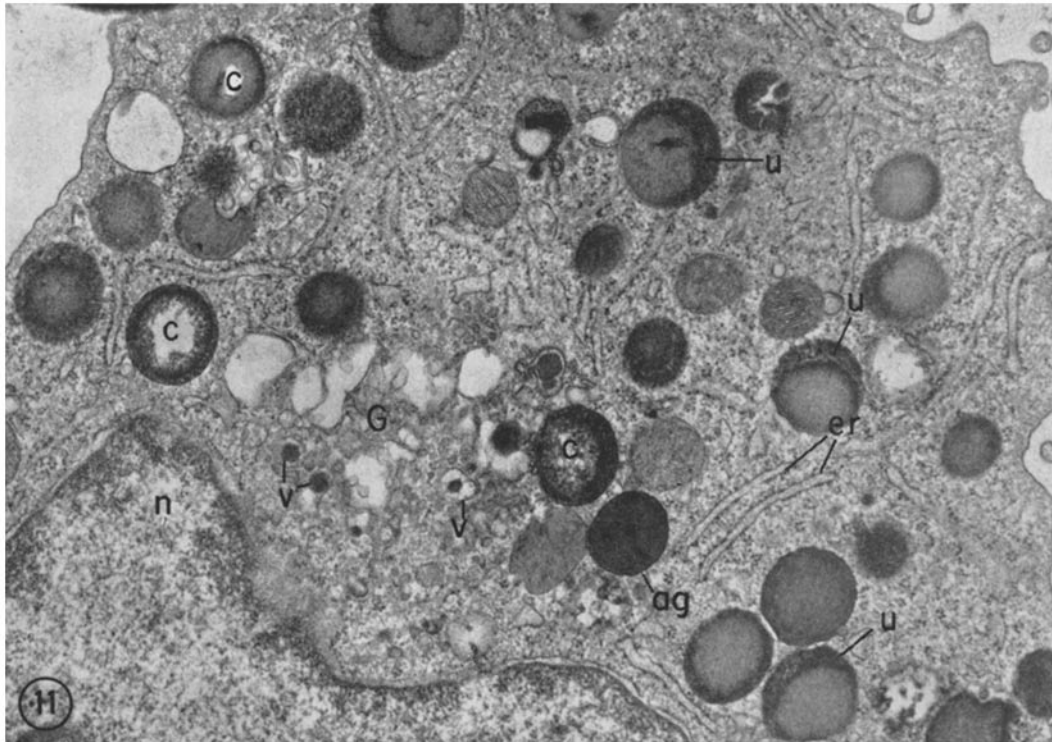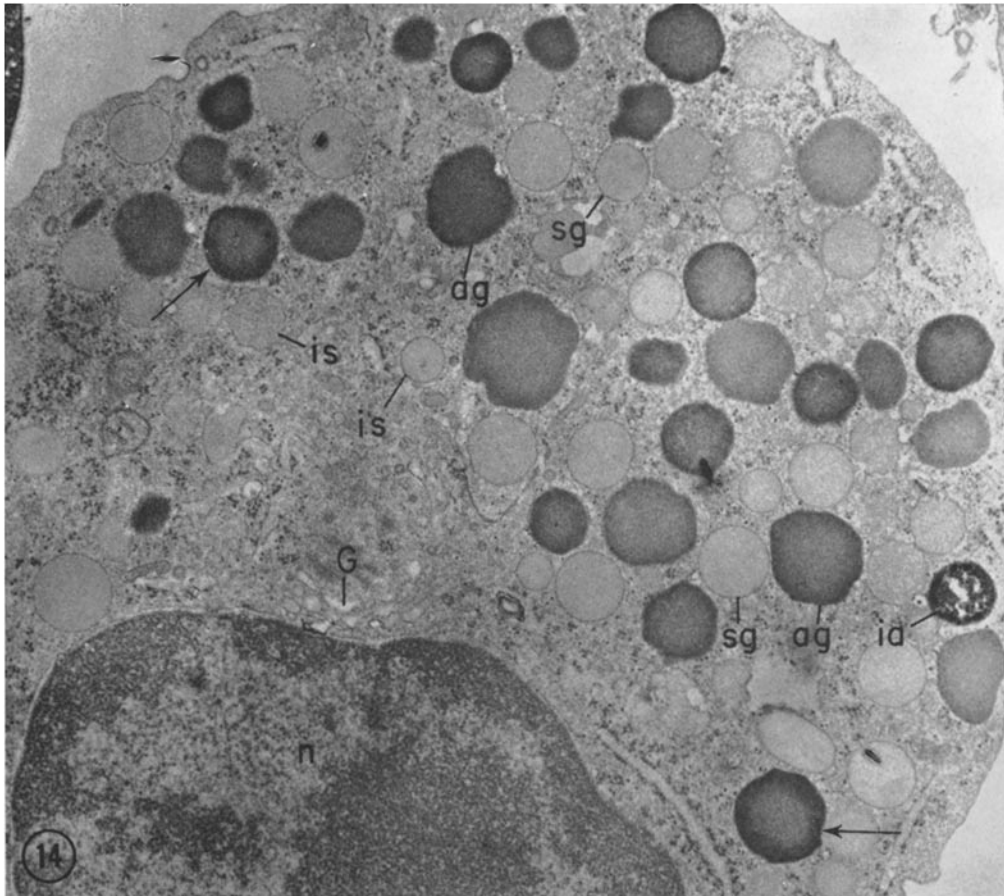
FIGURE 14 PMN myelocyte reacted for peroxidase, showing a reaction in azurophil (ag) but not in specific (sg) granules. Most of the azurophils are mature and appear uniformly dense owing to the presence of reaction product throughout the granules. In a few, the centers appear lighter (arrows). A single immature azurophil granule (ia) with more flocculent deposits of reaction product is also seen to the right. Several immature specific granules (is), not reactive for this enzyme, are indicated near the Golgi complex (G). Specimen preparation as for Fig. 11. × 18,000.

reaction product were finer and less chipping was evident. However, a diffuse precipitate was frequently seen in the background (Figs. 15 a and 18).

LOCALIZATION: Reaction product was seen in (a) immature azurophil granules, and (b) occasional ER and Golgi cisternae of the progranulocyte. Only some of the immature azurophil granules in a given cell contained reaction product (Fig. 15). The nucleoid form was most frequently reactive and contained the heaviest deposits (Figs. 16 and 17). Reaction product was usually restricted to that part of the granule exclusive of the core

material. Only individual cisternae of the ER in a given progranulocyte contained deposits (Fig. 15). Within the Golgi complex, reaction product was usually limited to the innermost cisternae (Fig. 15 a). The reaction in ER and Golgi elements was recognizable only in sections treated with $(NH_4)_2S$. In untreated specimens reaction product is apparently lost by chipping, as already has been mentioned. No reaction was seen in mature azurophil granules, in specific granules (Fig. 18), or in ER and Golgi cisternae of the myelocyte.

CONTROLS: In specimens incubated without substrate, reaction product was occasionally seen in
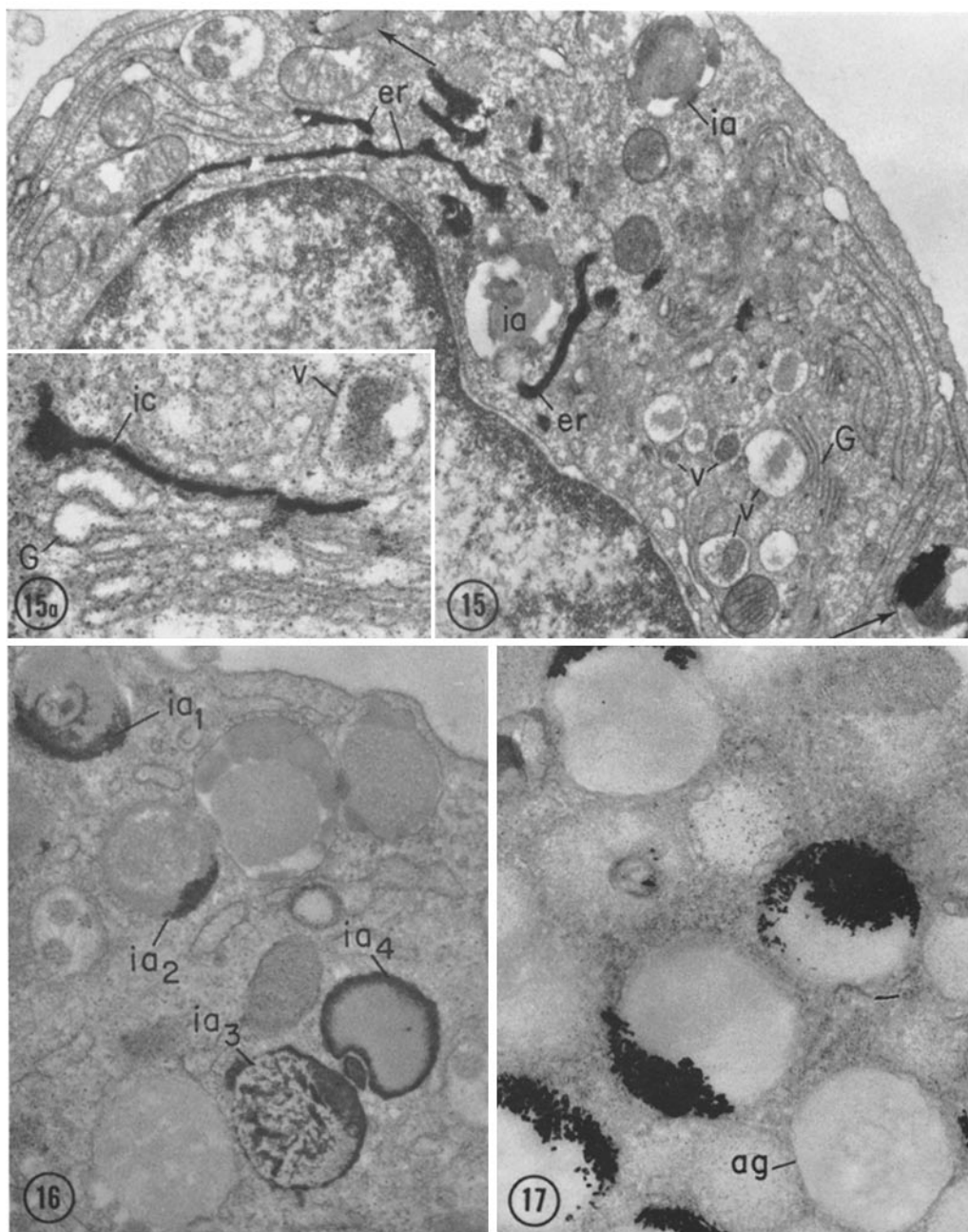
FIGURES 15–17 Developing PMN from preparations incubated for arylsulfatase. Fig. 15 shows deposits of reaction product in segments of some ER (er) cisternae and in a few of the immature azurophil granules (arrows) in a progranulocyte. The Golgi complex (G) and many of the forming azurophil granules (v and ia) are not reactive in this cell. The inset (Fig. 15 a) depicts reaction product in the innermost of the stacked Golgi cisternae (ic) of another progranulocyte. Note that fine particles cover the background in this preparation which was treated in block with (NH₄)₂S. Fig. 16 shows a group of immature azurophil granules from a progranulocyte. Four (ia₁–ia₄) contain reaction product; the remaining granules in the field, all of which are immature azurophils, do not. Fig. 17 shows several nucleoid forms of immature azurophil granules each with a rim of reaction product. An adjacent mature azurophil granule (ag) is unreactive. Note that the lead sulfate reaction product seen in Fig. 17 is coarser than the lead sulfide seen in the remaining figures. Specimens fixed in glutaraldehyde, incubated for 60 min at 37° C in Goldfischer's medium (pH 5.5), postfixed in OsO₄, treated in block with uranyl acetate, and embedded in Araldite or Epon. All except that in Fig. 17 were treated with (NH₄)₂S after incubation. Sections (except Fig. 17) stained with lead citrate. Fig. 15, × 15,500; Fig. 15 a, × 56,000; Fig. 16, × 27,000; Fig. 17, × 48,000.
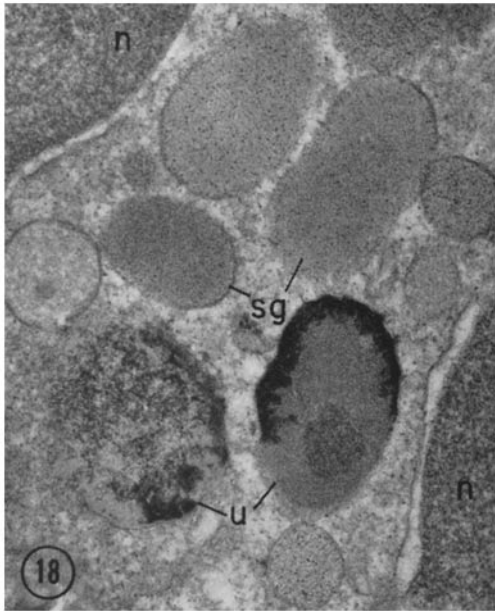
FIGURE 18 Group of granules in the cytoplasm of a PMN myelocyte incubated for arylsulfatase. Reaction product is seen in two nucleoid forms (u) of azurophil granules; specific granules (sg) are not reactive. Note that fine particles cover the background in this preparation which was treated in block with $(NH_4)_2S$. Specimen preparation as for Fig. 15. × 40,000.

small clumps in the perinuclear cisterna of progranulocytes, presumably on account of the presence of endogenous substrate.

5'-NUCLEOTIDASE, pH 4.0

GENERAL COMMENTS: The lead phosphate reaction product appeared in the form of fine particles aggregated in globules of about 80 A.

LOCALIZATION: The only sites which were reactive with this procedure were immature azurophil granules (Figs. 19–21). Reaction product was present in multilobulated forms (Figs. 19 and 20) and in nucleoids (Figs. 19 and 21), but it was not found in single-cored vacuoles (Fig. 19). None was seen in mature azurophil granules or in specific granules (Fig. 21).

An additional finding is that the core material of the azurophil nucleoids showed a concentric, lamellar "fingerprint" pattern with a regular 47 A spacing (see Fig. 28). This organization of the nucleoid was also evident in controls incubated

without substrate and in specimens reacted for acid phosphatase.

CONTROLS: The reaction was completely inhibited by the addition of 0.01 M NaF to the incubation medium.

ALKALINE PHOSPHATASE

GENERAL COMMENTS: Disruption of PMN structure was more extensive than with any other enzyme test; preservation was not improved by fixation for longer intervals (up to 4 hr). Of the methods tried, the calcium-lead method was in general the most satisfactory because the reaction was more extensive. With the direct lead method, morphological preservation was better, but the frequency with which reaction product was seen in Golgi elements (see below) was reduced. With the cadmium method, reaction product (cadmium phosphate) tended to chip out of the section in the same manner as described above for the lead sulfate obtained in the arylsulfatase test. All the methods produced a high background, i.e. there was a great deal of extraneous, presumably non-specific, precipitate scattered over the nucleus and cytoplasm.

LOCALIZATION: Deposits of reaction product were restricted to (a) immature specific granules, and (b) Golgi cisternae of some myelocytes. Within Golgi elements they occurred in all the cisternae (Fig. 22), but often were more concentrated in the outer ones. In immature specific granules, reaction product was frequently more concentrated at their periphery (Fig. 23).

CONTROLS: The reaction was completely inhibited by the addition of 0.001 M cysteine to the incubation medium and by the omission of substrate from the incubation medium.

ACID PHOSPHATASE

GENERAL COMMENTS: The lead phosphate reaction product appeared as needle-like crystals (30–40 A) finer than those obtained with 5'-nucleotidase or alkaline phosphatase. The distribution of reaction product was the same regardless of whether the original or modified (21) Gomori procedure was used or whether β-glycerophosphate or cytidine monophosphate was used as substrate. However, when β-glycerophosphate containing large quantities (∼25%) of the α isomer (Sigma Chemical Co.; grade III) was used, the reaction in ER cisternae of progranulocytes and myelocytes (see below) was more uniform and intense.
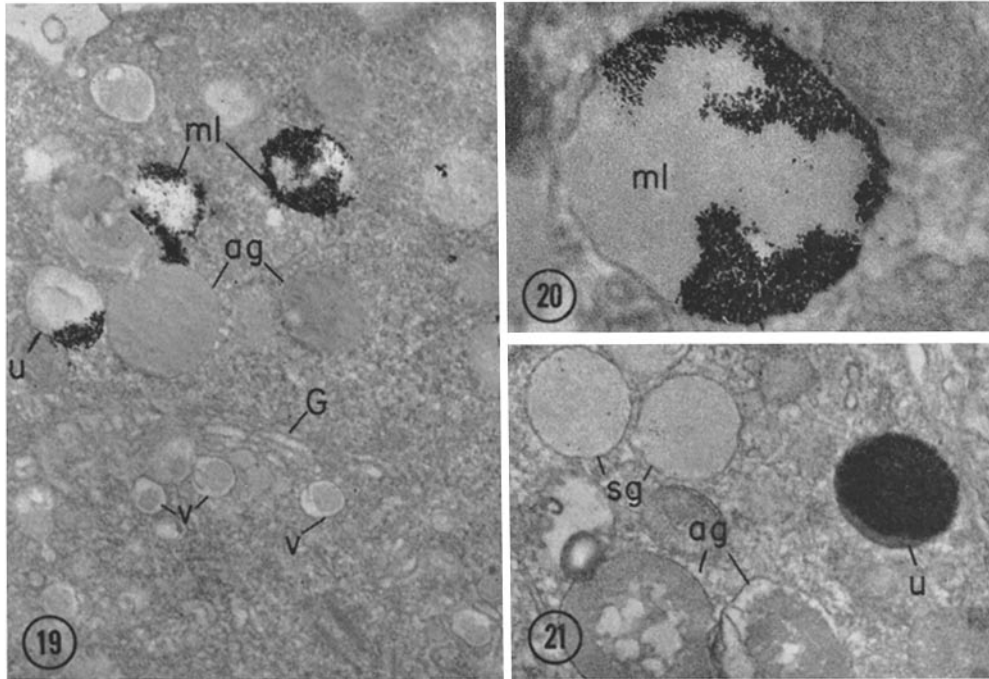
FIGURES 19–21 Small fields from developing PMN incubated for 5'-nucleotidase. In Fig. 19, part of a programulocyte is seen. Reaction product is present in some of the immature azurophil granules: several multilobulated forms (*ml*) and a nucleoid (*u*). No reaction is present in single-cored vacuoles (*v*), in Golgi cisternae (*G*), or in mature azurophil granules (*ag*). In Fig. 20, heavy deposits of reaction product are seen in an immature azurophil granule, filling the space between the multilobulated core material (*ml*) and the granule membrane. This is the earliest form of azurophil granule in which reaction product was seen. In Fig. 21, several azurophil (*ag*) and specific granules (*sg*) from a PMN myelocyte are shown. Only the nucleoid form (*u*) of azurophil granule contains reaction product. Specimens fixed in glutaraldehyde, incubated 90 min at 37° C in Wachstein-Meisel's medium with adenosine-5'-monophosphate at pH 4.0, postfixed in OsO₄, treated in block with uranyl acetate, and embedded in Araldite. The sections in Figs. 19 and 20 are unstained; that in Fig. 21 was stained with lead citrate. Fig. 19, × 21,000; Fig. 20, × 70,000; Fig. 21, × 39,000.

LOCALIZATION: Deposits were found within (*a*) immature azurophil granules, (*b*) ER and Golgi cisternae of progranulocytes, (*c*) immature specific granules, and (*d*) ER and Golgi cisternae of myelocytes. Among immature azurophil granules, all stages were found to contain heavy deposits (Figs. 24–26), but not all granules in a given field were reactive. As in the case of 5'-nucleotidase and arylsulfatase, reaction product was usually restricted to that part of the granule exclusive of the core material (Figs. 24 and 25). Reaction product was not seen in mature azurophil granules (Fig. 26). In progranulocytes, a fine sprinkling of lead phosphate was seen occasionally in ER cisternae, including the perinuclear cisterna (Fig. 27), and heavier deposits were seen in

Golgi cisternae. Frequently all of the stacked Golgi cisternae contained reaction product, but the innermost usually contained heavier deposits (Fig. 24).

Only some of the immature specific granules (Fig. 30) in myelocytes contained reaction product; fully condensed, mature specific granules were not reactive. The reaction in ER and Golgi cisternae of myelocytes was similar to that in progranulocytes except that in the Golgi complex the gradient was reversed; the heavier deposits occurred in the outermost cisternae (Fig. 29). This reaction in Golgi cisternae was seen throughout the myelocyte stage.

CONTROLS: In specimens incubated without substrate or with NaF added to the medium, no
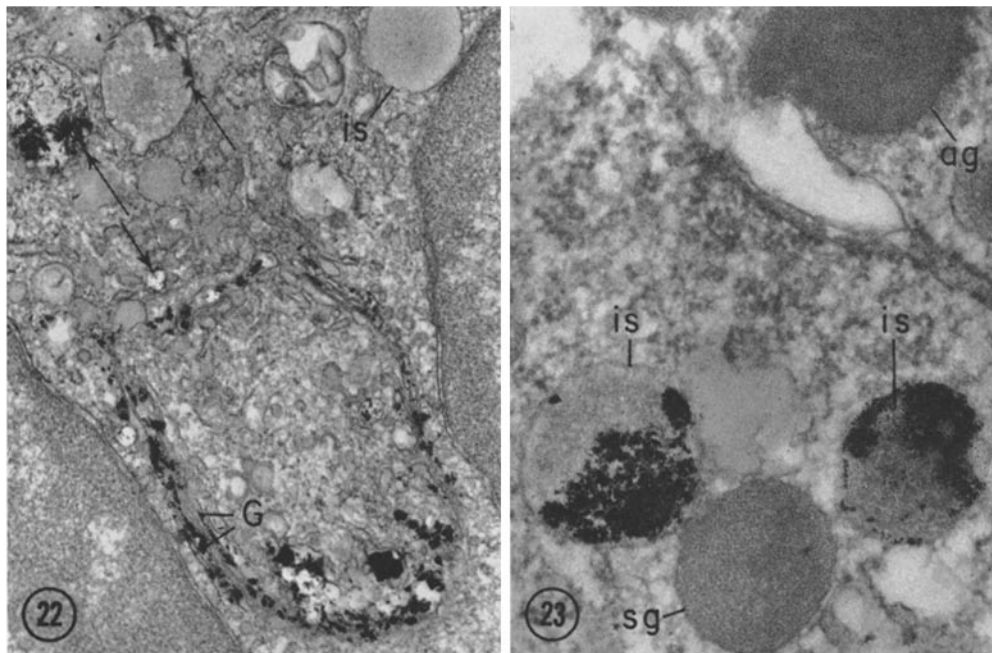
Figures 22 and 23 Alkaline phosphatase preparations of PMN myelocytes. In Fig. 22, reaction product is present in all of the Golgi cisternae (G) and in some of the immature specific granules (arrows). Other immature specific granules (is) are unreactive. In Fig. 23, reaction product is shown in two immature specific granules (is). The mature specific (sg) and a mature azurophil (ag) are not reactive. Specimen preparation as for Figs. 19–21, except that incubation was carried out in Gomori's medium at pH 9.2 and was followed by treatment with lead nitrate. Sections stained with lead citrate. Fig. 22, × 25,000; Fig. 23, × 62,000.

reaction product was seen in any of the above-mentioned sites. Addition of cysteine (an inhibitor of alkaline phosphatase) to the medium did not affect the reaction in either progranulocytes or myelocytes.

Special Treatments

None of the treatments produced a deposition of reaction product in mature granules. Several caused an increase in the intensity of the acid phosphatase reaction as evaluated by light microscopy as follows: (a) freezing and thawing six times, (b) sectioning on a cryostat or freezing microtome, (c) prolonging incubation times (up to 3 hr), and (d) adding 10% DMSO to the incubation medium. By electron microscopy it was apparent that this was due to an increase in the over-all reaction intensity and not to activation of fully-condensed mature granules. The first two procedures (both of which involved freezing the tissue) produced considerable cellular disruption, extraction of

azurophil granules, and a diffuse deposition of reaction product. Two treatments produced a decrease in intensity of the reaction by light microscopy, (a) addition of 0.5% Triton X-100 to the incubation medium, and (b) preincubation at pH 2.5. By electron microscopy it was apparent that with Triton X-100 there was no localization of reaction product; diffuse deposits covered the cells. With tissue preincubated at low pH, reaction product was localized at the sites described for untreated cells, but there was a marked decrease in the reaction. Both procedures produced extraction of azurophil granules. No effect on the reaction was noted at either the light or electron microscopic level with protamine or sparine. Finally, in incubations carried out with unfixed cells, no deposits of reaction product were seen.

DISCUSSION

The present findings confirm and extend our previous work (1) indicating that differences exist
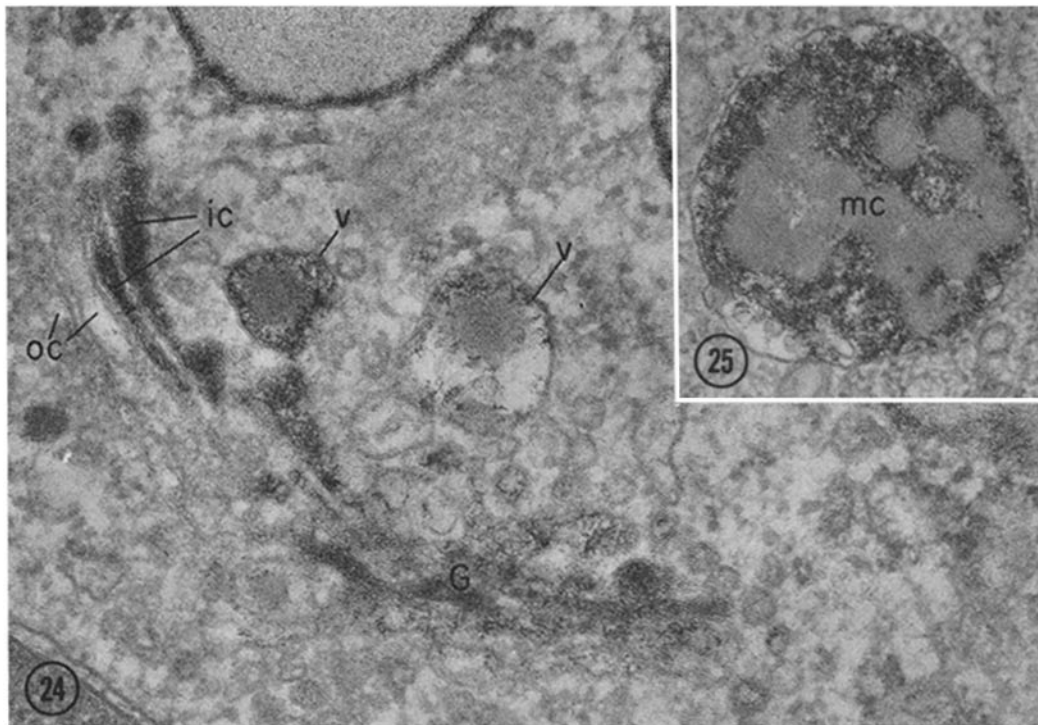
FIGURES 24 and 25   Acid phosphatase preparations of developing PMN. Fig. 24 shows the Golgi region of a PMN progranulocyte. Reaction product is present in several of the innermost cisternae (ic) and in early forming azurophil granules (v) located along the concave face of the Golgi complex. It is not seen in the outer Golgi cisterna (oc). Fig. 25 shows deposits of reaction product in a multicored aggregate (mc), a later stage of forming azurophil granule. The deposits of lead phosphate are concentrated between the core material (mc) and the membrane. Specimen preparation as for Fig. 19 except that incubation was carried out in modified Gomori's medium at pH 5.0. Sections stained with lead citrate. Fig. 24,   X 80,000; Fig. 25,   X 52,000.

in the enzyme composition of azurophil and specific PMN leukocyte granules. In the present study, azurophil granules were found to contain peroxidase and three lysosomal enzymes—acid phosphatase, arylsulfatase, and 5'-nucleotidase. All azurophils appeared to contain peroxidase, but the remaining enzymes were demonstrable only in a small proportion of immature granules. Hence the findings do not exclude the existence of further heterogeneity among azurophil granules in relation to these enzymes.

In accordance with the results obtained on smears, specific granules were found to contain alkaline phosphatase activity. In addition, however, these granules were found to contain phosphatasic activity at low pH. This latter activity does not appear to represent the acid tail of alkaline phosphatase, since it was not inhibited by

cysteine; moreover, it was sensitive to F^- and hence behaves like acid phosphatase. From the results obtained previously on smears, which showed that mature cells are less reactive than progranulocytes, it is concluded that the concentration of this enzyme in specific granules must be low, much lower than that in azurophils. The exact nature of this activity found in immature specific granules and in Golgi cisternae of myelocytes remains unknown, but it is reminiscent of a similar activity demonstrated with the Gomori acid phosphatase method in Golgi cisternae and in immature secretory granules in many other cells, e.g. parotid (22), anterior pituitary (4), exocrine (27) and endocrine (22) pancreas, adrenal medulla (28), neurosecretory (29), and Paneth's cells (22). In cells of the adrenal medulla, acid phosphatase activity has been found to be

associated with secretory granules *in vitro* as well as *in situ*; moreover, the activity present in secretory granule subfractions differs from that in lysosomal subfractions (30). Further information about the nature of the acid phosphatase activity in specific PMN granules and especially its relationship to the usual lysosomal acid phosphatase must await separation of this protein following separation of PMN granules. In this regard, however it is worth mentioning that the presence of two different acid phosphatases has already been reported (31) in rat PMN.

From the foregoing, it follows that, except for their content of myeloperoxidase, azurophil granules are comparable to lysosomes, whereas specific granules represent an entirely different secretion product which contains alkaline phosphatase, possibly antibacterial cationic proteins and/or lysozyme (see reference 1), and a phosphatase active at low pH, which at the moment is only poorly understood.

Results of previous work on localization of enzymes in PMN granules by cytochemistry and electron microscopy have been contradictory. Yamada (32) stressed homogeneity of peroxidase distribution in granules of developing rat PMN, and Miller (33) of acid and alkaline phosphatase distribution in PMN granules of rodent and rabbit marrow. On the other hand, Daems and van der Ploeg (34), Goldfischer et al. (35), and Enomoto and Kitani (36) reported that peroxidase was limited to large granules, probably corresponding to azurophil granules, in PMN from human blood.[1] Moreover, Wetzel et al. (20) noted heterogeneity of

---

[1] Our own observations indicate heterogeneity of peroxidase distribution among PMN granules in the species thus far examined (human, rat, cat, and guinea pig). In all cases, the first granules formed, which presumably represent azurophil granules, are

both acid and alkaline phosphatase activity among granules from PMN in rabbit bone marrow. These workers postulated (37) the existence of three granule types which they called "primary," "secondary," and "tertiary" granules, believed to be formed at early, middle, and late stages, respectively, of PMN development; they ascribed acid phosphatase activity to the first and third types, and alkaline phosphatase to the second. We found no evidence in this or in our earlier work (1–3, 38) for the existence of more than two types of granules. However, the existence of subpopulations of the two main types with different enzyme content cannot be ruled out because as already pointed out, except for peroxidase, only a portion of the granules of a given type were reactive with any of the tests.

Latency of Enzyme Activity in Mature PMN Granules

Our observations have brought out the fact that, when bone marrow is prepared for electron microscopy and cytochemistry, immature PMN granules are reactive with metal salt techniques, but mature granules of either type are not. Evidently during granule maturation, some alteration occurs in the permeability of the granule membrane or in the form of its content so that the enzymes therein cannot be demonstrated with methods of the Gomori type, although they are still demonstrable by azo dye or benzidine methods. In order to investigate this problem we carried out a number of procedures which had been successfully used by others to overcome latency of enzyme activity in lysosomes *in vitro* or *in situ*; these include freezing and thawing (39), prolonged incubation at 37° C (40,) incubation of unfixed

---

reactive for peroxidase, whereas the smaller granules formed later, presumably specifics, are not.

FIGURES 26–28 Fields from PMN progranulocytes incubated for acid phosphatase. Fig. 26 illustrates the localization of reaction product in late stages of forming azurophil granules. Heavy deposits are seen in a multilobulated form (*ml*) and in two nucleoids (*u*). Sparse deposits are seen in another nucleoid (*u′*). Fully condensed or mature azurophil granules (*ag*) are not reactive. Several other immature azurophil granules (*ia*) do not contain reaction product. Fig. 27 demonstrates reaction product in the rough-surfaced ER (*er*) and perinuclear cisterna (*pn*) of a progranulocyte. Fig. 28 depicts part of a nucleoid form of azurophil granule. A concentric fingerprint pattern with a spacing of 47 A is evident in the core material. This type of pattern is also seen in controls incubated with lead, but the peripheral deposits (arrows) are absent. Specimen preparation as for Figs. 24 and 25. Fig. 26, × 50,000; Fig. 27, × 60,000; Fig. 28, × 180,000.
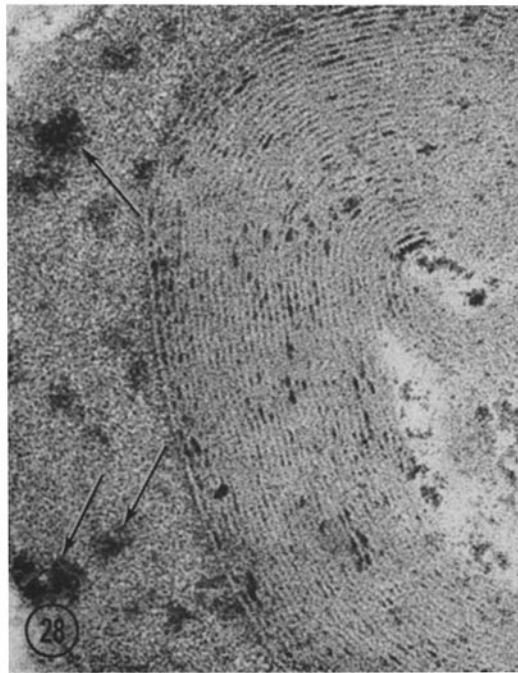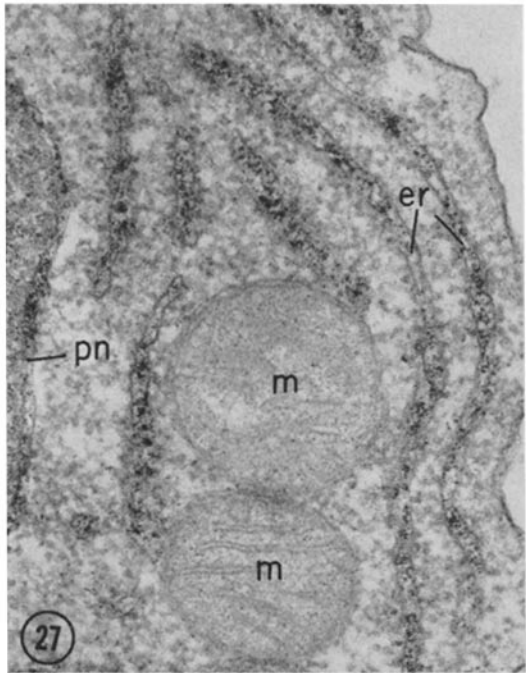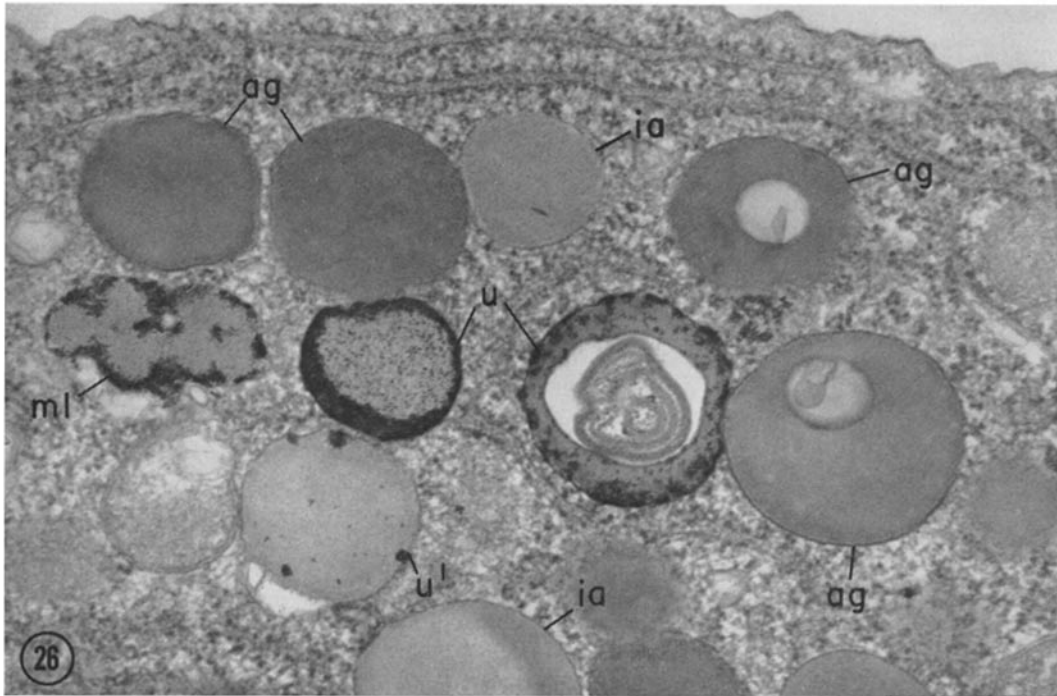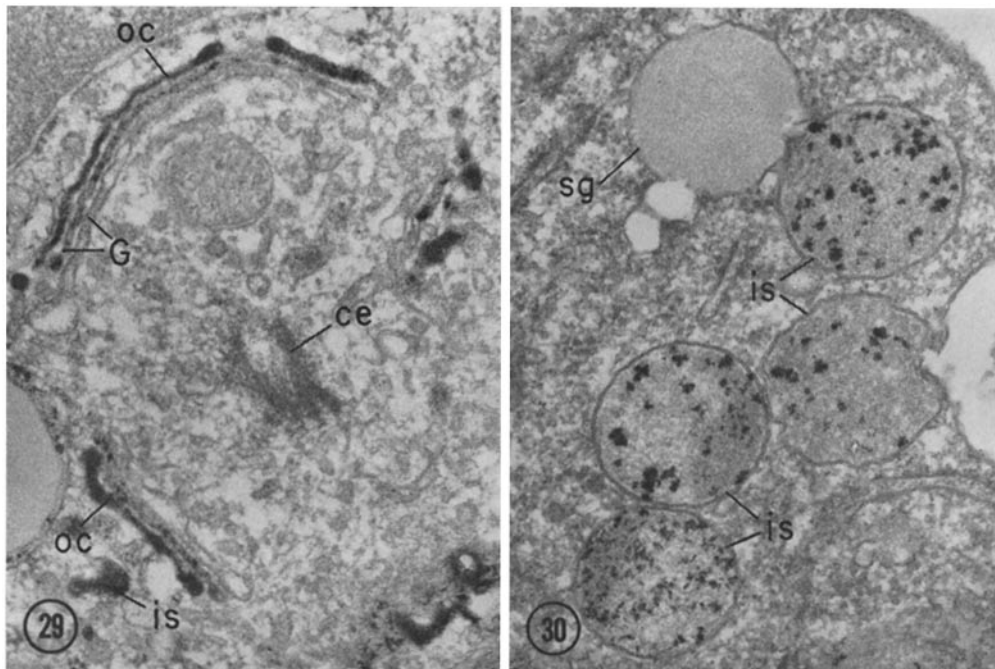
FIGURE 29 Golgi region of a PMN myelocyte incubated for acid phosphatase. Lead phosphate deposits are seen within all the Golgi cisternae (G), but they are most concentrated in the outermost cisternae (oc) along the convex Golgi face. Deposits are also seen in elements believed to be immature specific granules (is), located near the outer cisternae. × 43,000.

FIGURE 30 Group of specific granules in the cytoplasm of a PMN myelocyte reacted for acid phosphatase. The immature forms (is) contain scattered deposits of lead phosphate. No deposits are seen in an adjacent mature granule (sg) with more compact content. × 45,000.

For Figs. 29 and 30 specimen preparation was as for Figs. 24 and 25.

cells (41), and treatment with Triton X-100 (39), sparine (42), protamine (42), or DMSO (43, 44). However, no cytochemical reaction was detected in mature granules after any of these procedures. Two treatments (freezing and thawing and Triton X-100) may have solubilized the enzyme since extraction of azurophil granules and diffuse deposition of reaction product were seen. The only type of preparation in which latency was overcome or reduced was the smear extensively studied previously (1).

A lack of uniform reactivity of PMN granules with the Gomori acid phosphatase technique was mentioned by Miller (33), Enomoto and Kitani (36), White (45), and Seeman and Palade (46). These last workers studied the same phenomenon extensively in eosinophil granules. The difference in reactivity of lysosomes with the Gomori and dye

methods for acid phosphatase has been mentioned previously in other tissues (47), but this is neither widely known nor understood. From the general standpoint, it is of interest to note that the behavior of PMN granules, which represent primary lysosomes or storage granules, is different in this respect from that of secondary lysosomes for which the Gomori technique has been widely used as a lysosomal marker.[2] The observations of Weber

[2] Moreover, in peritoneal exudates (produced by a single injection of endotoxin), we found that the phagocytic vacuoles of macrophages were usually reactive for acid phosphatase, whereas PMN granules were not. When heat-killed Escherichia coli were mixed with the exudates for 10 min, many phagocytic vacuoles in PMN were reactive, but the surrounding granules in the same cell were not.

(48) on macrophage granules and especially of Seeman and Palade (46) on eosinophil granules suggest that a similar phenomenon may apply to primary lysosomes (storage granules) in some other sites.

Comments on Sites of Enzyme Localization in Cell Organelles

In addition to the findings on enzyme content of PMN granules, several more general observations were made on enzyme localization in developing PMN.

(a) Within immature granules, reaction product was typically confined to the lighter, peripheral portion of the forming granule and was not usually seen in the central dense material (i.e. cores or nucleoids).

(b) Reaction product was found in ER cisternae of developing PMN with two of the enzyme tests (arylsulfatase and acid phosphatase) and in Golgi cisternae with three tests (the same, plus alkaline phosphatase).

(c) In those instances where reaction product was found in the Golgi complex, deposits were most concentrated in cisternae located along the face involved in granule formation (inner cisternae of progranulocytes and outer cisternae of myelocytes).

Available evidence (see references 22, 49–51) suggests that lysosomal enzymes, like pancreatic secretory proteins (52), are synthesized on ribosomes, transferred to the cavities of the ER, and concentrated within elements of the Golgi complex.

The evidence obtained in this study is in keeping with these assumptions, for a given enzyme was localized within ER and Golgi elements only during the period of formation of the granule type in which it was also localized (see Table I). The fact that ER and Golgi reactions were not obtained with all the tests could be due to lack of sufficient concentration of enzyme[3] to be detected by available procedures or to technical factors involved in fixation, incubation, or embedding.[4]

[3] Our own observations as well as those of Ackerman (53) in cat marrow indicate that peroxidase reaction product is demonstrable in all ER and Golgi cisternae of PMN progranulocytes. Similar findings also pertain to the eosinophilic myelocytes of the rabbit and rat (32, 54).

[4] For instance, with modifications that presumably affect the quality of the reaction product, such as $(NH_4)_2S$ treatment of arylsulfatase preparations (14), additional reactive sites may be detected.

REFERENCES

1. BAINTON, D. F., and M. G. FARQUHAR. 1968. Differences in enzyme content of azurophil and specific granules of PMN leukocytes. I. Histochemical staining of bone marrow smears. *J. Cell Biol.* 39:286.

2. BAINTON, D. F., and M. G. FARQUHAR. 1966. Enzyme composition of polymorphonuclear leukocyte granules. *J. Cell Biol.* 31:8A.

3. BAINTON, D. F., and M. G. FARQUHAR. 1966. Origin of granules in polymorphonuclear leukocytes. Two types derived from opposite faces of the Golgi complex in developing granulocytes. *J. Cell Biol.* 28:277.

4. SMITH, R. E., and M. G. FARQUHAR. 1966. Lysosome function in the regulation of the secretory process in cells of the anterior pituitary gland. *J. Cell Biol.* 31:319.

5. SABATINI, D. D., K. BENSCH, and R. J. BARRNETT.

1963. Cytochemistry and electron microscopy. The preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation. *J. Cell Biol.* 17:19.

6. BAKER, J. R. 1946. The histochemical recognition of lipine. *Quart. J. Microscop. Sci.* 87:441.

7. PEASE, D. C. 1964. Histological Techniques for Electron Microscopy. Academic Press Inc., New York and London. 2nd edition. 51.

8. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J. Cell Biol.* 27: 137A.

9. TORACK, R. M. 1965. Adenosine triphosphatase activity in rat brain following differential fixation with formaldehyde, glutaraldehyde, and hydroxyadipaldehyde. *J. Histochem. Cytochem.* 13:191.

10. KARNOVSKY, M. J. 1965. Vesicular transport of exogenous peroxidase across capillary endothelium into the T system of muscle. *J. Cell Biol.* 27:49A.

11. GRAHAM, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney; ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* 14:291.

12. GOLDFISCHER, S. 1965. The cytochemical demonstration of lysosomal aryl sulfatase activity by light and electron microscopy. *J. Histochem. Cytochem.* 13:520.

13. HOPSU-HAVU, V. K., A. U. ARSTILA, H. J. HELMINEN, H. O. KALIMO, and G. G. GLENNER. 1967. Improvements in the method for the electron microscopic localization of arylsulphatase activity. *Histochemie.* 8:54.

14. HOLTZMAN, E., and R. DOMINITZ. 1968. Cytochemical studies of lysosomes, Golgi apparatus and endoplasmic reticulum in secretion and protein uptake by adrenal medulla cells of the rat. *J. Histochem. Cytochem.* 16:320.

15. WACHSTEIN, M., and E. MEISEL. 1957. Histochemistry of hepatic phosphatases at a physiologic pH. *Am. J. Clin. Pathol.* 27:13.

16. GOMORI, G. 1953. Enzymes. *In* Microscopic Histochemistry, Principles and Practice. University of Chicago Press, Chicago, Ill. 137.

17. HUGON, J., and M. BORGERS. 1966. A direct lead method for the electron microscopic visualization of alkaline phosphatase activity. *J. Histochem. Cytochem.* 14:429.

18. MIZUTANI, A., and R. J. BARRNETT. 1965. Fine structural demonstration of phosphatase activity at pH 9. *Nature.* 206:1001.

19. MOLNAR, J. 1952. The use of rhodizonate in enzymatic histochemistry. *Stain Technol.* 27:221.

20. WETZEL, B. K., S. S. SPICER, and R. G. HORN. 1967. Fine structural localization of acid and alkaline phosphatases in cells of rabbit blood and bone marrow. *J. Histochem. Cytochem.* 15:311.

21. BARKA, T., and P. J. ANDERSON. 1962. Histochemical methods for acid phosphatase using hexazonium pararosanilin as coupler. *J. Histochem. Cytochem.* 10:741.

22. NOVIKOFF, A. B. 1963. Lysosomes in the physiology and pathology of cells: contributions of staining methods. *In* Ciba Foundation Symposium on Lysosomes. A. V. S. de Reuck and M. P. Cameron, editors. Little, Brown and Company, Boston. 36.

23. MALAMED, S. 1963. Use of a microcentrifuge for preparation of isolated mitochondria and cell suspensions for electron microscopy. *J. Cell Biol.* 18:696.

24. FARQUHAR, M. G., and G. E. PALADE. 1965. Cell junctions in amphibian skin. *J. Cell Biol.* 26:263.

25. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* 9:409.

26. VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* 25:407.

27. SOBEL, H. J., and E. AVRIN. 1965. Localization of acid phosphatase activity in rat pancreatic acinar cells: a light and electron microscopic study. *J. Histochem. Cytochem.* 13:301.

28. GREENBERG, R., G. FALK, and C. A. KOLEN. 1966. Localization of acid phosphatase in the stimulated adrenal medullary cell of the rat. *J. Cell Biol.* 31:42A.

29. OSINCHAK, J. 1964. Electron microscopic localization of acid phosphatase and thiamine pyrophosphatase activity in hypothalamic neurosecretory cells of the rat. *J. Cell Biol.* 21:35.

30. GREENBERG, R., G. FALK, and C. A. KOLEN. 1967. Origin of acid phosphatase associated with adrenal medullary granules. *Federation Proc.* 26:493.

31. YU, B. P., F. A. KUMMEROW, and T. NISHIDA. 1966. Acid phosphatases of rat polymorphonuclear leucocytes. *Proc. Soc. Exptl. Biol. Med.* 122:1045.

32. YAMADA, E. 1966. Electron microscopy of the peroxidase in the granular leucocytes of rat bone marrow. *Arch. Hist. Japon.* 27:131.

33. MILLER, F. 1966. Electron microscopic cytochemistry of leucocyte granules. *In* Sixth International Congress for Electron Microscopy, Kyoto, Japan, 1966. R. Uyeda, editor. Maruzen Co. Ltd., Tokyo. 2:71.

34. DAEMS, W. T., and M. VAN DER PLOEG. 1966. On the heterogeneity of human neutrophilic leucocyte granules. *In* Sixth International Congress for Electron Microscopy, Kyoto, Japan, 1966. R. Uyeda, editor. Maruzen Co. Ltd., Tokyo. 2:83.

35. GOLDFISCHER, S., H. VILLAVERDE, and R. FORSCHIRM. 1966. The demonstration of acid hydrolase, thermostable reduced diphosphopyridine nucleotide tetrazolium reductase and peroxidase activities in human lipofuscin pigment granules. *J. Histochem. Cytochem.* 14:641.

36. ENOMOTO, T., and T. KITANI. 1966. Electron microscopic studies on peroxidase and acid phosphatase reaction in human leukocytes (In normal and leukemic cells and on the phagocytosis). *Acta Haematol. Japon.* 29:554.

37. WETZEL, B. K., R. G. HORN, and S. S. SPICER. 1967. Fine structural studies on the develop-

ment of heterophil, eosinophil, and basophil granulocytes in rabbits. *Lab. Invest.* **16**:349.

38. BAINTON, D. F., and M. G. FARQUHAR. 1965. Origin and nature of polymorphonuclear leukocyte granules. *J. Cell Biol.* **27**:6A.

39. DE DUVE, C. 1963. The lysosome concept. *In* Ciba Foundation Symposium on Lysosomes. A. V. S. de Reuck and M. P. Cameron, editors. Little, Brown and Company, Boston. 1.

40. SHIBKO, S., J. PANGBORN, and A. L. TAPPEL. 1965. Studies on the release of lysosomal enzymes from kidney lysosomes. *J. Cell Biol.* **25**:479.

41. BITENSKY, L. 1963. The reversible activation of lysosomes in normal cells and the effects of pathological conditions. *In* Ciba Foundation Symposium on Lysosomes. A. V. S. de Reuck and M. P. Cameron, editors. Little, Brown and Company, Boston. 362.

42. KOENIG, H., D. GAINES, T. McDONALD, J. SCOTT, and R. GRAY. 1964. Isolation and properties of brain lysosomes. *Federation Proc.* **23**:383.

43. MISCH, D. W. 1965. The penetration of lysosomes by dimethyl sulfoxide. *J. Cell Biol.* **27**:67A.

44. MISCH, D. W., and M. S. MISCH. 1967. Dimethyl sulfoxide: activation of lysosomes *in vitro*. *Proc. Natl. Acad. Sci. U. S.* **58**:2462.

45. WHITE, J. G. 1966. The Chediak-Higashi syndrome: a possible lysosomal disease. *Blood.* **28**:143.

46. SEEMAN, P. M., and G. E. PALADE. 1967. Acid phosphatase localization in rabbit eosinophils. *J. Cell Biol.* **34**:745.

47. HOLT, S. J., and A. B. NOVIKOFF. 1963. Discussion. *In* Ciba Foundation Symposium on Lysosomes. A. V. S. de Reuck and M. P. Cameron, editors. Little, Brown and Company, Boston. 375.

48. WEBER, R. 1964. Ultrastructural changes in regressing tail muscles of *Xenopus* larvae at metamorphosis. *J. Cell Biol.* **22**:481.

49. COHN, Z. A., M. E. FEDORKO, and J. G. HIRSCH. 1966. The in vitro differentiation of mononuclear phagocytes. V. The formation of macrophage lysosomes. *J. Exptl. Med.* **123**:757.

50. FEDORKO, M. E., and J. G. HIRSCH. 1966. Cytoplasmic granule formation in myelocytes. An electron microscope radioautographic study on the mechanism of formation of cytoplasmic granules in rabbit heterophilic myelocytes. *J. Cell Biol.* **29**:307.

51. DE DUVE, C., and R. WATTIAUX. 1966. Functions of lysosomes. *Ann. Rev. Physiol.* **28**:435.

52. PALADE, G. E. 1966. Structure and function at the cellular level. *J. Am. Med. Assoc.* **198**:815.

53. ACKERMAN, G. 1968. Contributions of the nuclear envelope, endoplasmic reticulum, and Golgi to the formation of peroxidase and lysosomal (azurophil) granules during neutrophil granulopoiesis. *Anat. Record.* **160**:304.

54. BAINTON, D. F., and M. G. FARQUHAR. 1967. Segregation and packaging of granule enzymes in eosinophils. *J. Cell Biol.* **35**:6A.